Among the various well characterized glycoprotein hormones, lutropin, follitropin, thyrotropin, and human chorionic gonadotropin, the detailed carbohydrate structure thus far has been determined only of hCG\(^2\) (1, 2). While hCG has both \(\beta\)- and \(\alpha\)-subunits, the \(\beta\)-subunit is the only one having a carbohydrate unit. The presence of this sugar in the \(\beta\)-subunit was identified as a sulfate ester. The present communication describes the carbohydrate structures of all three \(\beta\)-glycosidically linked carbohydrates in ovine LH, two in the \(\alpha\)-subunit and one in the \(\beta\)-subunit. The structural characterization has been carried out on each of the three glycopeptides and the oligosaccharide obtained by alkaline sodium borohydride treatment of oLH and individual subunits. The techniques employed for the structural elucidation involved methylation, periodate oxidation, deamination, acetylation, and hydrolysis with exoglycosidases. A preliminary report of this work has been made earlier (11, 12).

**EXPERIMENTAL PROCEDURES**

**Materials**

Frozen sheep pituitaries were purchased from Esco Endocrine Research Supply Co., San Mateo, CA. N-hydroxysuccinimide, and \(\alpha\)-amino-\(\beta\)-guanidinopropionic acid hydrochloride were obtained from Pierce Chemical Co. Dithioerythritol, iodoacetamide, glucosamine/HCl, galactosamine/HCl, and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin were procured from Sigma. Acetone, methyl iodide, pyridine, and dimethyl sulfoxide were purchased from Fisher. MeSO\(_4\) was dried by stirring overnight with calcium chloride followed by distillation under vacuum. The dry MeSO\(_4\) was stored over Type 5A molecular sieves, 8 to 12 mesh (J. T. Baker Chemical Co). Pyridine was dried by distillation over sodium hydroxide All other chemicals were of Baker analytical grade reagents from J. T. Baker Chemical Co. Bio-Gel P-2 and P-4 (both 100 to 200 mesh), and analytical grade ion exchange resins, AG 50W-X4 (H\(^+\), 200 to 400 mesh, and AG 3-X4A (CI\(^-\)), 30 to 50 mesh were purchased from Bio-Rad Laboratories. The latter was converted to acetate form prior to use. Amberlite IRA 400 and Amberlite MB 3 (H\(^+\), OH\(^-\)) were obtained from Mallinckrodt. All Sephadexes, including carboxymethyl-Sephadex and LH Sephadexes, were products of Pharmacia Fine Chemicals, Piscataway, NJ.

Highly purified glycosidases, \(\beta\)-galactosidase, \(\beta\)-mannosidase, and \(\beta\)-N-acetylglucosaminidase were prepared in this laboratory (13) from a commercial preparation of Aspergillus niger. \(\beta\)-N-Acetylglucosaminidase was also isolated from Turbatrix aceti (14). Jack bean meal \(\alpha\)-d-mannosidase was a commercial preparation purchased from Boehringer Mannheim. Certain disaccharides employed for the preparation of the methylated reference compounds were available from Dr. S. Wallin, Department of Chemistry, University of New York at Buffalo, Amherst Campus, Buffalo, New York 14260.

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1 To whom all correspondence regarding this manuscript should be addressed.

2 The abbreviations used are: hCG, human chorionic gonadotropin; oLH, ovine lutropin; oLH-\(\alpha\) and oLH-\(\beta\), the \(\alpha\)- and \(\beta\)-subunits of oLH; FSH, follitropin; TSH, thyrotropin; Man, \(\alpha\)-D-mannose; Gal, \(\alpha\)-D-galactose; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; Man-\(\beta\), \(\beta\)-D-mannoside; GlcNAc-\(\beta\), N-acetyl-D-glucosaminidase; 2,3,4,6-anhMan-\(\beta\), 2,5-anhydro-D-mannoside; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; MeSO\(_4\), dimethyl sulfoxide; GLC/MS, gas-liquid chromatography and mass spectrometry; TLC, thin layer chromatography.
laboratory. These included 2-O-, 3-O-, 4-O-, and 6-O-α-d-mannopyrano-syl-β-mannosides and 3-O-, 4-O-, and 6-O-α-acetamido-2-deoxy-α- d-mannopyranosyl-β-glucoses (15). Chitotetraose and α-glyco-peptide were prepared according to the published procedures (16, 17). 3-O-Methyl-2-deoxy-2-N-methylacetamido-ß-D-galactopyranoside was synthesized by methylation of 2-acetamido-ß-D-galactopyranose, using 3% sodium borohydride and acetic acid, as described above. The excess borohydride was destroyed with a drop of glacial acetic acid and the boric acid was removed by repeated evaporation with 5% acetic acid. Two more additions of TPCK-trypsin (0.25% each) were carried out on Cel 300-10 (Machery-Nagel) thin layer chromatography plates (20 × 20 cm) in pyridine-acetic acid/water (10:100:890), pH 3.7. Prior to use for chromatography, plates were baked in an oven at 110 °C and maintaining at that temperature. Under these conditions, the 2,5-anhydro sugars were well separated from each other as well as from other neutral sugars including mannose, galactose, and fucose. Combined gas-liquid chromatography-mass spectrometry was performed on the Hewlett-Packard Model 5992A system, fitted with a glass column (2 mm inner diameter × 200 cm) packed with OV-225.

Alditol acetates. GLC was performed by a Varian Gas Chromatograph Series 3700 either on a column packed with 3% OV-225 at 170 °C isothermally for 5 min followed by an increase in temperature of 5 °C/min to 190 °C and holding at that temperature, or on 3% ECNSS-M column, programmed for 120–190 °C with a linear increase of 2 °C/min. The relative detector response of 0.5 was calculated for glycerol.

Preparation of Ovine Luteinizing Hormone and Its Subunits—Ovine luteinizing hormone was purified from frozen sheep pituitaries by the procedure of Sherwood et al. (32). The LH activity was extracted from sheep pituitaries by ethylacetate-chloroform, followed by precipitation with 25% saturated aqueous ammonium sulfate, chromatography on a column of CM-Sephadex, and gel filtration on a column of Sephadex G-100. The LH activity during the purification was monitored either by in vitro receptor-binding assay or by radioimmunoassay. The LH-a and LH-ß subunits were separated by disc gel electrophoresis. The purity of the LH-a subunit was estimated by GLC for glycerol. The purity of the LH-a subunit was determined by the micro-barium chloranilate procedure (31).

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subjected successively to acetylation and hydrolysis as described by Stellner et al. (40). The methylated sugars thus released were reduced with sodium borohydride and acetylated with acetic anhydride/pyridine (1:1) (v/v). The alditol acetates of the partially methylated sugars were analyzed on a Hewlett Packard Model 5992A GC/MS system equipped with a membrane separator. Separation of partially methylated hexitol and hexosaminitol acetates was achieved on columns (0.02 cm inner diameter × 200 cm) packed with 3% OV-225 on Supelcoport, 80 to 100 mesh, and 3% OV-17 on Chromosorb WHP, 80 to 100 mesh, respectively. The sugar derivatives were identified by comparison of their retention times (41) relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, or 3,4,6-tri-O-methyl-1,5-di-O-acetyl-2-deoxy-2-N-methylacetamido-D-glucitol, as well as by mass fragmentogram. The conditions used for mass spectrometry were as follows: mass range, 40 to 298; ion-source temperature, 140 ± 10 °C; ionizing electron energy, 70 eV. The chromatograms were recorded with respect to total ions as well as 117 m/e (116 m/e for hexosaminitol derivatives). The bar spectrum and the relative abundance of pertinent mass ions for each derivative was retrieved from the computer at the end of each run.

Periodate Oxidation and Smith Degradation (42)—Pronase glycopeptides, intact oLH, or the individual subunits (0.1 to 1.0 nmol) were oxidized with 1.0 to 5.0 ml of 0.08 M NaIO4 in 0.05 M acetic buffer, pH 4.5 ± 0.1 °C for 60 h, in the dark. The reaction mixture was adjusted to pH 8.0 with 1 M NaOH. A 2 M sodium borohydride solution was added to a final concentration of 0.2 M sodium borohydride. Reduction was completed for 24 h at 4 °C. Excess borohydride was destroyed by the addition of a few drops of glacial acetic acid. The reaction mixture was lyophilized, dissolved in 1 mL of 0.002 M acetic acid, and desalted on a column (1.5 × 100 cm) of Sephadex G-25 (fine). Glycopeptide or glycoprotein fractions were pooled, lyophilized, and dissolved in a known volume of water. Appropriate aliquots were taken for amino acid, hexosamine, and neutral sugar analyses. The remaining sample was subjected to partial acid hydrolysis in 1 mL of 0.5 N HCl for 48 h at room temperature, or with 1 ml of 0.05 N H2SO4 for 90 min at 80 °C in an oven. The products of partial acid hydrolysis of periodate oxidized-reduced glycopeptides were fractionated on a column (1.5 × 90 cm) of Sephadex G-25. The periodate oxidized-reduced intact hormone or subunits were partially hydrolyzed with 0.05 N H2SO4 as above and the hydrolysate was neutralized with barium carbonate. After the removal of the precipitate of BaSO4 by centrifugation, the supernatant was passed through a column (10 × 10 cm) of SP-Sephadex C-50. The unretarded low molecular weight oligosaccharides, fraction eluted from the column with water, was lyophilized and further purified by preparative TLC in Solvents F and G. The sample held to the SP-Sephadex was eluted with 0.5 M NaCl, lyophilized, and desalted by gel filtration on a column (1.5 × 90 cm) of Sephadex G-25 (fine).

Nitrous Acid Deamination of Oligosaccharides—The nitrous acid deamination of the N-acetylated oligosaccharides was performed by a slightly modified procedure of Horon et al. (43). In a typical experiment about 350 nmol of the oligosaccharide in 350 ml of water were mixed with an equal volume of a 5% solution of sodium nitrite and 33% acetic acid and kept at room temperature for 6 h. The reaction mixture was deionized by passing through successive columns (1 × 5 cm) of Amberlite IRA 400 (H+ form) and Bio-Rad AG 50 W-X8, and the effluent and washings were lyophilized without delay. The lyophilized material was reduced with NaBH4 and/or NaB3H4 and fractionated by paper chromatography in Solvent A and/or by high voltage paper electrophoresis in 1% borate buffer.

Enzymatic Hydrolysis of oLH Glycopeptides—Glycopeptides (30 to 50 nmol) in 100 μl of 0.05 M citrate/phosphate buffer, pH 4.8, were treated with a mixture of β-N-acetylgalactosaminidase (0.5 to 1.0 unit) and β-galactosidase (0.1 to 0.2 unit). The incubation was continued for 5 days at 37 °C in an atmosphere of nitrogen. The sample was layered with toluene by adding a drop of solvent to avoid any bacterial growth. N-Acetylgalactosamine released was determined by the method of Reiser et al. (27). A part of the reaction mixture was passed through mixed bed resin (Amberlite MB 3), eluted with water, reduced with NaBH4 and acetylated and the sugars released by enzymes were analyzed by GLC. Similarly, various acetylation and deamination fragments were subjected to enzymatic hydrolysis.

RESULTS

Amino Acid and Carbohydrate Compositions of oLH, oLH-α, and oLH-β Subunits—oLH and subunits employed in these studies were homogeneous by electrophoresis in polyacrylamide gel (data not shown) and their amino acid compositions were in close agreement with those reported (3, 6, 32). The carbohydrate compositions of oLH, oLH-α, and oLH-β, determined by GLC as alditol acetates for neutral sugars (29) and by an amino acid analyzer for hexosamines (1) are given in Table I. Since there are three asparagine-linked oligosaccharides in oLH, each carbohydrate unit must contain on the average, 3 residues of mannose, 3 to 4 residues of N-acetylgalactosamine, 1 residue of N-acetylglucosamine, 1 residue of N-acetylglucosamine, and 0 to 1 residue each of fucose and galactose. Sialic acid content, determined by Warren’s method (30) was negligible. The carbohydrate compositions of oLH-α and oLH-β were also found to be similar when calculated on the basis of 2 and 1 asparagine-linked oligosaccharides, respectively.

Preparation of Glycopeptides and Oligosaccharides—The glycopeptides were prepared from the reduced and S-carbamidomethyl oLH-α and oLH-β by tryptic digestion followed by the fractionation of the tryptic peptides and glycopeptides by gel filtration on Sephadex G-50 (superfine). Two major carbohydrate-containing peaks, preceded by a shoulder were obtained from oLH-α (Fig. 1, upper panel). Analytical high voltage paper electrophoresis at pH 1.8 showed that first major peak (pool 2) comprised of only one component (MGI = 0.3), while the second peak (pool 3) was a mixture of three ninhydrin-staining components (MGI = 0.48, 0.55, and 0.60) with electrophoretic mobilities relative to glycine as shown in the parentheses. The fastest moving spot (MGI = 0.66) stained for carbohydrate with periodate-ammoniacal silver nitrate. Additionally, when each spot was eluted and analyzed for amino acids and hexosamines, the two slow moving components were

| Table I | Carbohydrate compositions of oLH, oLH-α and oLH-β and their glycopeptides and oligosaccharids |

<table>
<thead>
<tr>
<th>Sugar</th>
<th>oLH</th>
<th>Oligosaccharide</th>
<th>oLH-α</th>
<th>oLH-β</th>
<th>βGP-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylglucosamine</td>
<td>10.1</td>
<td>2.5</td>
<td>7.1</td>
<td>3.2</td>
<td>3.0</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>2.9</td>
<td>1.0</td>
<td>1.8</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Mannose</td>
<td>7.3</td>
<td>2.8</td>
<td>6.4</td>
<td>3.1</td>
<td>3.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.1</td>
<td>0.4</td>
<td>0.9</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Fucose</td>
<td>1.4</td>
<td>0.5</td>
<td>0.7</td>
<td>0.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*a Not determined.

b Undetectable.
Carbohydrate Structure of oLH

Methylation Analysis—The three glycopeptides and the oligosaccharides were methylated by the procedure of Hako-mori (38), and were hydrolyzed, reduced, and acetylated essentially according to the procedure of Stellner et al. (40). The partially methylated alditol and hexosaminyl acetates were identified by comparison of their retention times relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-d-glucitol with the reported values (41), as well as by comparison with those of authentic standards. Linkage analysis and relative retention times of methylated sugars as alditol acetates are summarized in Table II. The results were further confirmed by GLC/MS analysis. Fragmentation patterns were compared with those reported by Jannson et al. (24) for partially methylated alditol acetate standards of neutral sugars and by Stellner et al. (40) for partially methylated acetylated hexosaminyl standards and also with the known methylated sugars prepared in this laboratory. The three glycopeptides and oligosaccharide yielded almost identical methylated derivatives as given below: 2,3,4-tri-O-methyl-2-fucose (0.1 to 0.3 residue), 2,3,4,6-tetra-O-methyl-d-galactose (0.1 to 0.3 residue), 3,4,6-tri-O-methyl-d-mannose (1.6 to 2.0 residues), 2,4-di-O-methyl-p-mannose (1.0 residue), 2-deoxy-3,4,6-tri-O-methyl-2-N-methylacetaldo-d-glucose (0.9 to 1.0 residue), 2-deoxy-3,6-di-O-methyl-2-N-methylacetaldo-d-glucose (1.5 to 1.9 residues), 2-deoxy-3-O-methyl-2-N-methylacetaldo-d-glucose (0.1 to 0.4 residue), and 2-deoxy-3,6-di-O-methyl-2-N-methylacetaldo-d-glucose (0.5 to 0.9 residue), the latter being quantitated only approximately because this peak was not well resolved from that of 2-deoxy-3,6-di-O-methyl-2-N-methylacetaldo-d-glucose. The permethylated oligosaccharide also revealed the presence of 0.4 residue of 1,3,5-tri-O-methyl-2-N-methylacetaldo-d-glucitol. The relative number of residues of various methylated derivatives in each glycopeptide are described in Table III.

Methylation analysis of the glycopeptides after partial acid hydrolysis with 0.5 N H<sub>2</sub>SO<sub>4</sub> in a boiling water bath resulted in the loss of 2-deoxy-3-O-methyl-2-N-methylacetaldo-d-glucose, 2,3,4-tri-O-methyl-fucose, and 2-deoxy-3,6-di-O-methyl-2-N-methylacetaldo-d-galactose, and the appearance of 2-deoxy-3,4,6-tri-O-methyl-2-N-methylacetaldo-d-galactose. The data clearly indicate that N-acetylglucosamine and N-acetylgalactosamine were substituted at C-1 and C-6 or C-1 and C-4 positions by fucose or some other acid-labile group.

Smith Degradation (42)—The treatment of all the three

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found to be devoid of any hexosamine. The shoulder was a mixture of two glycopeptides (M<sub>RG</sub> 0.13 and M<sub>RG</sub> 0.3). Based on the amino acid compositions, the two major peaks from oLH-α consisted of glycopeptides containing amino acid residues 56 to 67 and 80 to 95, respectively (3, 5). The shoulder contained the former glycopeptide except that it was made up of amino acid residues 50 to 67, formed by the incomplete cleavage between Lys<sub>m</sub>-Asn<sub>m</sub> (3, 5) due to steric hindrance by carbohydrate at Asn<sub>mg</sub>. The gel filtration of the tryptic digest of the reduced and S-carbamidomethyl oLH-P on Sephadex G-50 (Fig. 1, lower panel) yielded a single glycopeptide containing amino residues 7 to 20 (4, 6) as determined by amino acid analysis. Three tryptic glycopeptides (residues 56 to 67 and 80 to 95, respectively (3, 5)) thus obtained were digested extensively by pronase to degrade the peptide chains. The pronase-digested tryptic glycopeptides were designated as aGP-1, aGP-2, and βGP-3, respectively.

The oligosaccharides from oLH, oLH-α, and oLH-β were prepared by alkaline hydrolysis of the hormone or its subunits in the presence of NaB<sub>H</sub> (36). A sample of 1.5 mg of the intact hormone (45 mg) yielded 3.5 mg of the oligosaccharides, whereas 60 mg and 315 mg of the oligosaccharides were obtained from 0.5 mg (7.5 mg) each of oLH-α and oLH-β, respectively. The yield of the oligosaccharides thus obtained was 70 to 80% on the basis of 3, 2, and 1 asparaginyl oligosaccharides in oLH, oLH-α, and oLH-β, respectively.

Carbohydrate Compositions of Asparaginyl Glycopeptides aGP-1, aGP-2, and βGP-3, and the Oligosaccharide—The carbohydrate compositions of the three glycopeptides are described in Table I. Apparently, the compositions of all three glycopeptides were nearly identical with an average molar ratio of N-acetylglucosamine, N-acetylgalactosamine, mannose, galactose, and fucose as aspartic acid residue to be 3.2:1.0:2.9:0.4:0.5. The carbohydrate compositions of the oligosaccharides from oLH, oLH-α, or oLH-β were also similar to those of the three glycopeptides. Based on 1 residue of N-acetylglucosamine, the molar composition of the oligosaccharide in N-acetylglucosamine (2.6), N-acetylgalactosamine (1.0), N-acetylglucosaminidol (0.5), mannose (2.8), galactose (0.4), and fucose (0.5). The value for N-acetylgalactosaminidol was lower than expected, although the total amount of N-acetylglucosamine and N-acetylgalactosaminidol was 3.1 mol which was similar to that present in the glycopeptides.

Reducing Termini of the Oligosaccharides—In order to identify the sugar at the reducing terminus, the oligosaccharides obtained from either intact hormone or individual subunits by alkaline-NaB<sub>H</sub> hydrolysis (36) were analyzed for hexosamines and hexosaminidol by amino acid analyzer using buffer system described by Cheng and Boat (26). This dual buffer system has the advantage over single buffer system described by Weber and Winderz (25) that it improves significantly the resolution between glucosaminidol and galactosaminidol. The hexose and hexosaminidol analysis under these conditions showed only glucosamine, galactosamine, and glucosaminidol indicating that N-acetylgalactosaminidol residue was at the reducing termini of the oligosaccharides.

Methylation Analysis—The three tryptic glycopeptides and the oligosaccharides were methylated by the procedure of Hako-mori (38), and were hydrolyzed, reduced, and acetylated essentially according to the procedure of Stellner et al. (40). The partially methylated alditol and hexosaminidol acetates were identified by comparison of their retention times relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-d-glucitol with the reported values (41), as well as by comparison with those of authentic standards. Linkage analysis and relative retention times of methylated sugars as alditol acetates are summarized in Table II. The results were further confirmed by GLC/MS analysis. Fragmentation patterns were compared with those reported by Jannson et al. (24) for partially methylated alditol acetate standards of neutral sugars and by Stellner et al. (40) for partially methylated acetylated hexosaminidol standards and also with the known methylated sugars prepared in this laboratory. The three glycopeptides and oligosaccharide yielded almost identical methylated derivatives as given below: 2,3,4-tri-O-methyl-2-fucose (0.1 to 0.3 residue), 2,3,4,6-tetra-O-methyl-d-galactose (0.1 to 0.3 residue), 3,4,6-tri-O-methyl-d-mannose (1.6 to 2.0 residues), 2,4-di-O-methyl-p-mannose (1.0 residue), 2-deoxy-3,4,6-tri-O-methyl-2-N-methylacetaldo-d-glucose (0.9 to 1.0 residue), 2-deoxy-3,6-di-O-methyl-2-N-methylacetaldo-d-glucose (1.5 to 1.9 residues), 2-deoxy-3-O-methyl-2-N-methylacetaldo-d-glucose (0.1 to 0.4 residue), and 2-deoxy-3,6-di-O-methyl-2-N-methylacetaldo-d-galactose (0.5 to 0.9 residue), the latter being quantitated only approximately because this peak was not well resolved from that of 2-deoxy-3,6-di-O-methyl-2-N-methylacetaldo-d-glucose. The permethylated oligosaccharide also revealed the presence of 0.4 residue of 1,3,5-tri-O-methyl-2-N-methylacetaldo-d-glucitol. The relative number of residues of various methylated derivatives in each glycopeptide are described in Table III.

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Smith Degradation (42)—The treatment of all the three
TABLE II
Linkage analysis—relative retention times of methylated sugars as their alditol acetates obtained from methylated N-glycopeptides of oLH-α and oLH-β

Neutral sugars with respect to 2,3,4,6-tetra-O-methyl-d-glucitol and amino sugars with respect to 2-deoxy-3,4,6-tri-O-methyl-2-N-methylacetamido-d-glucitol.

<table>
<thead>
<tr>
<th>Methylated sugar</th>
<th>Position substituted</th>
<th>αGP-1</th>
<th>αGP-2</th>
<th>βGP-3</th>
<th>Literature</th>
<th>Reference compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>2,3,4-Tri-O-methyl</td>
<td>1-O</td>
<td>0.60</td>
<td>0.60</td>
<td>0.59</td>
<td>0.58</td>
</tr>
<tr>
<td>Galactose</td>
<td>2,3,4,6-Tetra-O-methyl</td>
<td>1-O</td>
<td>1.18</td>
<td>1.19</td>
<td>1.19</td>
<td>1.19^a</td>
</tr>
<tr>
<td>Mannose</td>
<td>3,4,6-Tri-O-methyl</td>
<td>1,2-Di-O-</td>
<td>1.82</td>
<td>1.81</td>
<td>1.81</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td>2,4-Di-O-methyl</td>
<td>1,3,6-Tri-O</td>
<td>4.28</td>
<td>4.27</td>
<td>4.28</td>
<td>4.51</td>
</tr>
<tr>
<td>2-Deoxy-2-N-methylacetaliglucose</td>
<td>3,4,6-Tri-O-methyl</td>
<td>1-O</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>3,6-Di-O-methyl</td>
<td>1,4-Di-O-</td>
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<td>3-O-Methyl</td>
<td>1,4,6-Tri-O</td>
<td>2.54</td>
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<tr>
<td>2-Deoxy-2-N-methylacetaligalactose</td>
<td>3,6-Di-O-methyl</td>
<td>1,4-Di-O-</td>
<td>1.58</td>
<td>1.58</td>
<td>1.58</td>
<td>1.59</td>
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</table>

Molar ratios of partially methylated alditol acetates obtained from the permethylated N-glycopeptides of oLH-α and oLH-β and oLH-oligosaccharide

<table>
<thead>
<tr>
<th>Methylated sugar</th>
<th>Position substituted</th>
<th>αGP-1</th>
<th>αGP-2</th>
<th>βGP-3</th>
<th>Oligosaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>2,3,4-Tri-O-methyl</td>
<td>1-O</td>
<td>0.2</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>2,3,4,6-Tetra-O-methyl</td>
<td>1-O</td>
<td>0.5</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Mannose</td>
<td>3,4,6-Tri-O-methyl</td>
<td>1,2-di-O-</td>
<td>2.0</td>
<td>1.8</td>
<td>2.1</td>
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<tr>
<td></td>
<td>2,4-Di-O-methyl</td>
<td>1,3,6-tri-O-</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2-Deoxy-2-N-methylacetaliglucose</td>
<td>3,4,6-Tri-O-methyl</td>
<td>1-O</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
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<td>3,6-Di-O-methyl</td>
<td>1,4-di-O-</td>
<td>1.6</td>
<td>1.9</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>2-O-Methyl</td>
<td>1,4,6-tri-O</td>
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<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>2-Deoxy-2-N-methylacetaligalactose</td>
<td>3,6-Di-O-methyl</td>
<td>1,4-di-O-</td>
<td>0.4</td>
<td>0.7</td>
<td>0.5</td>
</tr>
</tbody>
</table>

^a Calculated for ‘neutral’ sugars by assuming 2,4-di-O-methyl-d-mannitol as 1.0 and for amino sugars assuming all methylated derivatives of N-acetylgalactosamine to be 3.0.

glycopeptides with 8- to 10-fold excess of NaIO₄ at 4 °C for 60 h caused the destruction of 2 of the 3 residues of mannose, and all of galactose and fucose. Both N-acetylgalactosamine and N-acetylgalactosaminodeoxylactosamine survived the first cycle of Smith degradation (Table IV). The destruction of galactose, fucose, and 2 of the 3 residues of mannose is in agreement with the methylation data. The resistance to periodate oxidation of the nonreducing terminal N-acetylgalactosamine can not be reconciled with the methylation data which indicate all three glycopeptides containing approximately 0.9 to 1.0 residue of 2-deoxy-3,4,6-tri-O-methyl-2-N-methylacetamido-d-glucose.

In order to determine whether the discrepancy in the results by two methods was due to incomplete oxidation of N-acetylgalactosamine by periodate oxidation under the conditions used, Smith degradation was carried out under stronger conditions such as the oxidation at 4 °C for 144 h or at 40 °C for 60 h. This also failed to destroy the nonreducing terminal N-acetylgalactosamine. To eliminate the possibility that the aldehyde groups of the oxidized mannose residues form hemiacetals with the hydroxyl groups of the unoxidized residues of N-acetylgalactosamine, the periodate oxidized-reduced glycopeptide was again subjected to oxidation and reduction under the same conditions. N-Acetylgalactosamine survived the second oxidation. The treatment of the glycopeptides with 2 M NH₄OH for 4 h at room temperature prior to periodate oxidation also failed to oxidize N-acetylgalactosamine. This rules out the possibility of an alkali-labile substituent at either C-3 or C-4 position of the nonreducing terminal N-acetylgalactosamine. Such a substituent, if present, would be removed by nucleophilic attack of dimethyl sulfoxide anion during methylolation, giving 2-deoxy-3,4,6-tri-O-methyl-2-N-methylacetamido-d-glucose indicating the terminal position of N-acetylgalactosamine.

Efforts to separate the products of mild acid hydrolysis of the periodate oxidized-reduced glycopeptide by either gel filtration on Sephadex G-25, or ion exchange chromatography on a short column of Dowex 50-H⁺ were unsuccessful. Consequently, the periodate oxidation of the intact hormone or individual subunits was attempted in order to facilitate the separation of the Smith degradation products.

Periodate oxidation of the intact hormone or individual subunits also resulted in the complete destruction of galactose and fucose and two-thirds of the mannose residues (Table V). After mild acid hydrolysis (0.05 N H₂SO₄, 80 °C for 90 min) of
the periodate-oxidized oLH, oLH-α, and oLH-β, the products were fractionated into a low molecular weight oligosaccharide fraction and a glycopeptide fraction by SP-Sephadex chromatography. The former was further purified by TLC in Solvent F and G in which it migrated with N-acetylgalactosaminyl glyceraldehyde. Apparently, this low molecular weight fraction was derived from the peripheral sugar chains by the cleavage of the oxidized 1,2-linked mannose residues. The oligosaccharide fraction was reduced with NaBH₄/NaBH₄₅₃, hydrolyzed, and analyzed by paper chromatography in Solvent B. Only glycerol was detected as a radioactive component. Sugar analysis of the reduced oligosaccharide showed the presence of glycerol, N-acetylgalactosamine, and N-acetylgalactosamine in an equimolar ratio (Table V). Periodate oxidation of the oligosaccharide resulted in the destruction of 74% N-acetylgalactosamine and 66% N-acetylgalactosaminylgalactosamine. The data indicate that the small molecular weight oligosaccharide was made up of a mixture of N-acetylgalactosaminyl glyceraldehyde and N-acetylgalactosaminyl glyceraldehyde. It appears that the substituent at C-4 position of N-acetylgalactosamine was mostly removed during hydrolysis of the periodate oxidized-reduced oligosaccharide.

The residual glycopeptide, which eluted from SP-Sephadex with 0.5 M NaCl, was desalted by gel filtration on Sephadex G-25. Each glycopeptide thus obtained from oLH, oLH-α, and oLH-β was composed of 2 residues of N-acetylgalactosaminyl residue of mannose. A small amount of N-acetylgalactosamine present was due to incomplete hydrolysis of the oxidized-reduced glycopeptides (Table V). Treatment of this material with A. niger β-mannosidase resulted in the release of 45 to 55% of mannose. Further digestion of the β-mannosidase-treated material with T. aceti β-N-acetylgalactosaminidase liberated 40% of N-acetylgalactosamine. Therefore, the above evidence establishes the structure of the core glycopeptide to be Man β-GlcNAc β-GlcNAc—Asn.

**Deamination of oLH Oligosaccharide**—The oLH oligosaccharide obtained by alkaline sodium borohydride hydrolysis (36) was subjected to deamination by nitrous acid (43). After reducing 1% of the material with NaBH₄, the manuscript analysis was performed by TLC in Solvent A (Fig. 2). Stachyose, raffinose, sucrose, glucose, and 2,5-anhydro-1,3,6-tri-O-methyl-β-mannitol. Hydrolysis of the radioactive Fraction I after another cycle of Smith degradation gave only 2,5-anhydro-1,3,6-tri-O-methyl-β-mannitol. The data indicate that the small molecular weight oligosaccharide was made up of a mixture of N-acetylgalactosaminyl glyceraldehyde and N-acetylgalactosaminyl glyceraldehyde. It appears that the substituent at C-4 position of N-acetylgalactosamine was mostly removed during hydrolysis of the periodate oxidized-reduced oligosaccharide.

The residual glycopeptide, which eluted from SP-Sephadex with 0.5 M NaCl, was desalted by gel filtration on Sephadex G-25. Each glycopeptide thus obtained from oLH, oLH-α, and oLH-β was composed of 2 residues of N-acetylgalactosaminyl residue of mannose. A small amount of N-acetylgalactosamine present was due to incomplete hydrolysis of the oxidized-reduced glycopeptides (Table V). Treatment of this material with A. niger β-mannosidase resulted in the release of 45 to 55% of mannose. Further digestion of the β-mannosidase-treated material with T. aceti β-N-acetylgalactosaminidase liberated 40% of N-acetylgalactosamine. Therefore, the above evidence establishes the structure of the core glycopeptide to be Man β-GlcNAc β-GlcNAc—Asn.

**Table IV**

<table>
<thead>
<tr>
<th>Sugar*</th>
<th>Native After perio-</th>
<th>Native After perio-</th>
<th>Native After perio-</th>
<th>Native After perio-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>date oxidation</td>
<td>date oxidation</td>
<td>date oxidation</td>
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<tr>
<td>Mannose</td>
<td>3.1  1.2  3.0  1.2</td>
<td>3.1  1.2  3.0  1.2</td>
<td>3.1  1.2  3.0  1.2</td>
<td>3.1  1.2  3.0  1.2</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.5  0.0  0.1  0.0</td>
<td>0.5  0.0  0.1  0.0</td>
<td>0.5  0.0  0.1  0.0</td>
<td>0.5  0.0  0.1  0.0</td>
</tr>
<tr>
<td>Fucose</td>
<td>3.2  3.2  3.1  3.1</td>
<td>3.2  3.2  3.1  3.1</td>
<td>3.2  3.2  3.1  3.1</td>
<td>3.2  3.2  3.1  3.1</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>0.9  0.9  1.0  1.1</td>
<td>0.9  0.9  1.0  1.1</td>
<td>0.9  0.9  1.0  1.1</td>
<td>0.9  0.9  1.0  1.1</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>0.9  0.9  1.0  1.1</td>
<td>0.9  0.9  1.0  1.1</td>
<td>0.9  0.9  1.0  1.1</td>
<td>0.9  0.9  1.0  1.1</td>
</tr>
</tbody>
</table>

* Moles of monosaccharide per mol of aspartic acid.

**Table V**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Native*</th>
<th>OR*</th>
<th>High M⁺ glycopeptide</th>
<th>Low M⁺ fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>oLH</td>
<td>Glycerol</td>
<td>n.d.</td>
<td>n.d. 1.9</td>
<td>n.d. 1.9</td>
</tr>
<tr>
<td></td>
<td>Mannose</td>
<td>2.6</td>
<td>0.9  0.8</td>
<td>0  0</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>0.4</td>
<td>0  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td></td>
<td>Fucose</td>
<td>0.5</td>
<td>0  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td></td>
<td>N-Acetylgalactosamine</td>
<td>3.3</td>
<td>3.2  2.0</td>
<td>1.3  1.3</td>
</tr>
<tr>
<td></td>
<td>N-Acetylgalactosamine</td>
<td>1.0</td>
<td>1.0  0.1</td>
<td>1.0  1.0</td>
</tr>
<tr>
<td>oLH-α</td>
<td>Glycerol</td>
<td>n.d.</td>
<td>n.d. 1.9</td>
<td>n.d. 1.9</td>
</tr>
<tr>
<td></td>
<td>Mannose</td>
<td>3.2</td>
<td>1.1  1.0</td>
<td>1.0  1.0</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>0.3</td>
<td>0  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td></td>
<td>Fucose</td>
<td>0.3</td>
<td>0  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td></td>
<td>N-Acetylgalactosamine</td>
<td>3.4</td>
<td>3.0  2.0</td>
<td>1.1  1.1</td>
</tr>
<tr>
<td></td>
<td>N-Acetylgalactosamine</td>
<td>0.9</td>
<td>1.1  0.1</td>
<td>1.0  1.0</td>
</tr>
<tr>
<td>oLH-β</td>
<td>Glycerol</td>
<td>n.d.</td>
<td>n.d. 1.9</td>
<td>n.d. 1.9</td>
</tr>
<tr>
<td></td>
<td>Mannose</td>
<td>2.8</td>
<td>1.1  0.8</td>
<td>0  0</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
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<td>0  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td></td>
<td>Fucose</td>
<td>0.5</td>
<td>0  0  0</td>
<td>0  0  0</td>
</tr>
<tr>
<td></td>
<td>N-Acetylgalactosamine</td>
<td>3.2</td>
<td>3.1  2.0</td>
<td>1.2  1.2</td>
</tr>
<tr>
<td></td>
<td>N-Acetylgalactosamine</td>
<td>1.3</td>
<td>1.1  0.1</td>
<td>1.0  1.0</td>
</tr>
</tbody>
</table>

* Number of residues of sugar based on aspartic acid residues, 11 for oLH, 6 for oLH-α, and 5 for oLH-β, and also assuming 3, 2, and 1 asparagine-linked carbohydrate chains in oLH, oLH-α, and oLH-β, respectively.

**a** OR, periodate-oxidized, borohydride-reduced; ORH, periodate-oxidized, borohydride-reduced, acid-hydrolyzed.

**b** Obtained from SP-Sephadex by elution with 0.5 M NaCl and contains the innermost core trisaccharide.

**c** Untreated fragment from SP-Sephadex column and is derived from the outer branches of the carbohydrate unit.

* Not determined.

were 0.1:1.0:0.1:0.1:4.1:5.5, respectively. All fractions were analyzed for hexosamine after hydrolysis with 4 N HCl by an automatic amino acid analyzer. Except for Fraction I (Fig. 2), no other fraction contained any hexosamine. Fraction I was high molecular weight oligosaccharide arising from the incomplete deamination and, therefore, this fraction was not pursued any further. All other fractions were analyzed for their carbohydrate compositions (Table VI), and were further characterized as detailed below.

Fraction II (Rₕₒ, 0.44) migrated slightly faster than tetrasaccharide stachyose in Solvents A and C. It was found to contain, after hydrolysis with 2 N HCl for 2 h at 100 °C, 2,5-anhydrohamnitol and mannose in a ratio of 1:3:4 as determined by GLC as aldial acetates. To establish the reducing terminus, labeled Fraction II was hydrolyzed with 2 N HCl for 2 h at 100 °C and the hydrolysate was subjected to paper electrophoresis in borate buffer. Only one major radioactive peak with mobility identical with authentic standard 2,5-anhydrohamnitol was detected, indicating its presence at the reducing end. The tetrasaccharide after first cycle of Smith degradation yielded a disaccharide containing 2,5-anhydrohamnitol and mannose in a ratio of 1:0.1:1. This disaccharide after another cycle of Smith degradation gave only 2,5-anhydrohamnitol. Hydrolysis of the radioactive Fraction II with α-mannosidase yielded a product identical with that obtained after the first cycle of Smith degradation. Further digestion with β-mannosidase gave 2,5-anhydrohamnitol. Fraction II on methylation analysis gave 2 residues of 2,3,4,6-tetra-O-methyl-D-mannose, 1 residue of 2,4-di-O-methyl-D-mannose, and 0.3 residue of 2,5-anhydro-1,3,6-tri-O-methyl-D-mannitol. The 2,5-anhydro-1,3,6-tri-O-methyl-D-mannitol was identified by comparison of its retention time and mass spectrum with a
corresponding fraction obtained from the deamination products of α, 3-acid glycoprotein, and with the literature (45, 46). From the above data, the following structure was derived for the fraction II: Man—(Man—)-Man—2,5-anhydromannitol.

Fraction III (R_{g0} 0.62) co-migrated with raffinose on paper chromatography in Solvent A, and was composed of 2,5-anhydromannitol and mannose in a ratio of 1:2 as determined by GLC. Hydrolysis of the radioactive Fraction III with α-mannosidase yielded a product identical with that obtained after similar treatment of Fraction II. Further digestion with β-mannosidase or hydrolysis with 2 N HCl for 2 h at 100 °C resulted in the release of free 2,5-anhydromannitol. Consequently, Fraction III has the following structure: Man—α-Man—β-2,5-anhydromannitol.

Fraction IV (R_{g0} 0.96) was found to contain galactose, fucose, and 2,5-anhydromannitol in a molar ratio 0.6:0.5:1.0. The mobility of this fraction on paper in Solvent A was similar to sucrose. When the radioactive Fraction IV was hydrolyzed in 2 N HCl at 100 °C for 2 h, and the hydrolysate was analyzed by paper electrophoresis using borate buffer, 2,5-anhydromannitol was detected as the only radioactive component confirming its presence at the reducing end. Therefore, this fraction was a mixture of galactosyl-2,5-anhydromannitol and fucosyl-2,5-anhydromannitol.

Sugar analysis of Fraction V (R_{g0} 1.16) by GLC after acid hydrolysis showed that this fraction consisted of 2,5-anhydrotalitol. When the radioactive Fraction V was subjected to paper electrophoresis in borate buffer, it showed a mobility different from that of 2,5-anhydromannitol or 2,5-anhydrotalitol. However, after mild acid hydrolysis, it showed only 2,5-anhydrotalitol by electrophoresis in borate buffer. The identity of 2,5-anhydrotalitol was further confirmed after its elution from paper by GLC. Therefore, Fraction V has the structure X-2,5-anhydrotalitol, where X represents an acid-labile group. H-labeled and unlabeled X-2,5-anhydrotalitol were separated from the other deamination products by paper electrophoresis at pH 4.7. The X-2,5-anhydrotalitol-containing fraction migrated towards the anode, 12.5 cm relative to glucose. Since Whatman No. 1 paper was found to contain high background levels of sulfate, the X-2,5-anhydrotalitol was further purified by electrophoresis at pH 3.7 on a previously washed cellulose thin layer plate. H-labeled X-2,5-anhydrotalitol and 35SO_4^2- markers migrated 3.5 and 13 cm, respectively, on thin layer plates. After the elution of X-2,5-anhydrotalitol from the plate, it was hydrolyzed in 2 N HCl at 100 °C for 1 h. The HCl was removed under vacuum. Aliquots of 10% were analyzed for 2,5-anhydrotalitol by gas chromatography analysis of the alditol with arabinobutanol as an internal standard and for sulfate by the micro-barium chloranilate procedure of Spencer (50). Controls of the unhydrolyzed X-2,5-anhydrotalitol and the eluate from the corresponding blank strip of the thin layer plate after hydrolysis were also carried out. Approximately 1 residue of sulfate was found/mol of 2,5-anhydrotalitol.

Fraction VI (R_{g0} 1.36) was 2,5-anhydromannitol determined by GLC as well as by paper chromatography in Solvent A and paper electrophoresis in borate buffer. The structures of various deamination products are described in Fig. 3.

Acetylation of oLH Oligosaccharide—In order to determine whether N-acetylgalactosamine was present at the α1,3- or α1,6-linked mannose branch, the oligosaccharide, prepared by NaOH + NaBH_4 treatment of oLH, was subjected to acetylation in a mixture of acetic anhydride, glacial acetic acid, and concentrated sulfuric acid (10:10:1 v/v) at 40 °C for 16 h.

Table VI

<table>
<thead>
<tr>
<th>Composition (molar ratio)</th>
<th>Fraction</th>
<th>R_{g0}</th>
<th>Molar ratio</th>
<th>2,5-Anhydromannose</th>
<th>2,5-Anhydrotalitol</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mixture</td>
<td></td>
<td>0.44</td>
<td>1.0</td>
<td>1.4</td>
<td>0.3</td>
<td>3.0</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>0.62</td>
<td>0.1</td>
<td>1.0</td>
<td>Undect.</td>
<td>3.4</td>
<td>Undect.</td>
<td>Undect.</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>0.96</td>
<td>0.1</td>
<td>1.0</td>
<td>Undect.</td>
<td>2.0</td>
<td>Undect.</td>
<td>Undect.</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>1.16</td>
<td>0.4</td>
<td>Undect.</td>
<td>1.0</td>
<td>Undect.</td>
<td>Undect.</td>
<td>0.6</td>
</tr>
</tbody>
</table>

For fraction numbers, see Fig. 2.

Calculated on the basis of radioactivity relative to (Man)_{3}-2,5-anh.Man-H_2.

Number of sugar residues in various fragments by GLC assuming 2,5-anhydromannose to be 1, except for the total mixture which is based on 3 mannose residues.

Undetectable.
respectively. Fraction showed a single major radioactive peak with A-1
jected to paper chromatography deamination and was not investigated any further.

Fractions B to D were further purified by paper chromatography in Solv-0.63) on thin layer chromatography in Solvent F. Fractions B
oligosaccharide. The oligosaccharide fragments obtained after acetolysis were reduced with NaBH₄; glucose (Glc), and N-acetylglucosamin(olGlcNAc-H₂).

FIG. 3. The structures of various fragments obtained by the deamination of oLH oligosaccharide.

Fig. 4. Paper chromatography of the acetolysis products of oLH oligosaccharide. The oligosaccharide fragments obtained after acetolysis were reduced with NaBH₄, and/or NaBH₃ and were subjected to paper chromatography in Solvent C for 2 days. Insets B to D show the rechromatography of Fractions B, C, and D, respectively, in Solvent B for 2 days. The reference paper containing the labeled material was excised into 1-cm strips and counted. The reference compounds used were stachyose (Stachy); raffinose (Raf); lactose (Lac); glucose (Glc), and N-acetylglucosaminitol (GlcNAc-H₂).

Fig. 5. Separation scheme used for the fractionation of the acetolysis products. Per cent yield and the mobility on paper or TLC plate relative to glucose (Rcₜₒ) of each fraction are indicated.

**TABLE VII**

<table>
<thead>
<tr>
<th>Molar ratio of partially methylated alditol acetates derived from permethylated acetolysis products of oLH-oligosaccharide</th>
<th>Methylated sugar</th>
<th>Position substituted</th>
<th>Molar ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-methyl</td>
<td>1-O-</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>3,4,6-Tri-O-methyl</td>
<td>1.2-di-O-</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>2,4,6-Tri-O-methyl</td>
<td>1.3-di-O-</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>2,4-Di-O-methyl</td>
<td>1,3,6-tri-O-</td>
<td>1.5</td>
<td>0.7</td>
</tr>
<tr>
<td>2-Deoxy-2-N-methyl-oligosaccharide-0.3</td>
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</tr>
<tr>
<td>3,6-Di-O-methyl</td>
<td>1,4-di-O-</td>
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<td>0.4</td>
</tr>
<tr>
<td>3,4,6-Tri-O-methyl</td>
<td>1.1-di-O-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2-Deoxy-2-N-methyl-oligosaccharide-0.7</td>
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<td></td>
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</tr>
<tr>
<td>1,3,6-Tetra-O-methyl</td>
<td>4-O-</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* Calculated for neutral sugars on the basis of the total peak areas by GLC of 1,3- and 1,3,6-substituted mannose derivatives as 1.0, and for aminosugars on the basis of 1,4-di-substituted N-acetylglucosamine derivative as 1.0.

Not determined.

lyzed for neutral sugars by GLC (29) and for hexosamines by amino acid analyzer (1).

Acetolysis Fragment A-1 contained mannosae, N-acetylglucosamnine, and N-acetylglucosaminitol in a molar ratio of 2:0.1:3.0:7. On methylation, the oligosaccharide Fragment A-1 (Table VII) was found to consist of 2,3,4,6-tetra-O-methyl-d-mannose (0.6), 3,4,6-tri-O-methyl-d-mannose (0.9), 2,4,6-tri-O-methyl-d-mannose (0.3), 2,4-di-O-methyl-d-mannose (0.7), 2-deoxy-3,4,6-tri-O-methyl-2-N-methylacetamido-d-glucose (0.6), 2-deoxy-3,5-di-O-methyl-2-N-methylacetamido-d-glucose (1.0), and 2-deoxy-1,3,5,6-tetra-O-methyl-2-N-methylacetamido-d-glucitol. Fractions A-2 and A-3 also contained mannosae, N-acetylglucosamnine, and N-acetylglucosaminitol in molar ratios of 2:1:3:0.6 and 2:1:0.6, respectively. Methylation analysis of Fractions A-2 and A-3 showed a decrease in 2,4-di-O-methyl-d-mannose and a concomitant increase in 2,4,6-tri-O-methyl-d-mannose. Based on the above data, the following structure can be assigned to Fragments A-1, A-2, and A-3 GlcNAc-β-Man-α-(Man-α-)Man-β-GlcNAc-α-GlcNAc-H₂. Apparently, these fragments differ in their contents of 1,3,6-linked mannosyl residues. Since Fractions A-1 to A-3 have N-acetylglucosaminitol at the reducing end, and contain no N-acetylgalactosamine or N-acetylgalactosaminitol, they result from the degradation of the oligosaccharide from the nonreducing termini.
Carbohydrate analysis of Fraction B-1 showed that it was made up of mannose, glucosamine, and galactosamine. When radioactive Fraction B-1 was hydrolyzed with 2 N HCl for 2 h at 100 °C, and the hydrolysate subjected to paper chromatography in Solvent B, all the radioactivity was found to co-migrate with mannitol, indicating the presence of mannitol at the reducing end. On treatment with N-acetylglucosaminyl mannitol and N-acetylgalactosaminyl mannitol. Fraction C-1 was identified as free mannitol, while Fraction D-1 was a mixture of free N-acetylglucosaminyl and N-acetylgalactosaminyl mannitol. These monosaccharides are due to nonspecific degradation of the oligosaccharide during acetylation. It may be noted that the unidentified substituent on N-acetylgalactosamine was removed during acetylation.

Treatment of oLH Glycopeptides and Oligosaccharide with Exoglycosidases—The results of the exoglycosidase treatment of the three glycopeptides are summarized in Table VIII. Treatment of the glycopeptides directly with β-galactosidase from A. niger resulted in the liberation of 0.2 to 0.3 mol of galactose/mol of glycopeptide. None of the mannose present in either C-1 the three glycopeptides could be released by α-mannosidase alone or with β-galactosidase, but 0.2 to 0.8 residue of N-acetylgalactosamine was released from these glycopeptides when treated with either a mixture of β-galactosidase and P-N-acetylgalactosaminidase or β-N-acetylgalactosaminidase alone. No difference in the amount of N-acetylgalactosamine was released when β-N-acetylgalactosaminidase from A. niger or T. aceti or a mixture of both was used. A small amount of N-acetylgalactosamine was also released by β-N-acetylgalactosaminidase. This was probably because of the intrinsic β-N-acetylgalactosaminidase activity of the enzymes. These results indicate that β-galactose and N-acetylgalactosamine are located externally.

More detailed information on the anomeric linkages of the mannosyl residues was obtained from the structural characterization of the tetrasaccharide Man2(1,4)GalNAcGalNAc2. When radioactive tetrasaccharide (Fraction II from deamination) was incubated with α-mannosidase from jack bean meal, 2 mannosyl residues were removed and the residual oligosaccharide showed mobility as a disaccharide. Further digestion with β-mannosidase resulted in a radioactive peak with a mobility similar to that of authentic 2,5-anhydromannitol. Thus, the external 2 mannosyl residues are linked by a linkage while the internal mannose is linked to 2,5-anhydromannitol by a β linkage.

**DISCUSSION**

The carbohydrate compositions of the three asparagine-linked oligosaccharides of oLH are quite similar, with the exception of some minor differences in their galactose and fucose content probably due to microheterogeneity. Each carbohydrate unit was made of GlcNAc, GalNAc, Man, Gal, and Fuc. Based on the results of methylation, periodate oxidation, deamination, acetylation, and enzymatic hydrolysis with glycosidases, the following structure (Fig. 6) is proposed for the 3 carbohydrate units. The differences in the structures of the individual carbohydrate units reside in the varying amounts of peripheral galactosyl and fucosyl residues. The nonreducing terminal location of galactose, fucose, and N-acetylgalactosamine was established by methylation analysis. The release of all galactosyl residues and 0.2 to 0.8 residue of N-acetylgalactosamine by A. niger β-d-galactosidase and β-N-acetylgalactosaminidase, respectively, indicates further that these are located terminally. The terminal position of fucose was also shown by its susceptibility to hydrolysis under mild acid conditions. Periodate oxidation resulted in the destruction of galactose, fucose, and 2 of 3 mannose residues thus supporting the terminal positions of galactose and fucose and further indicating that the 2 mannose residues were linked by 1,2, 1,4, or 1,6 linkages. When the periodate oxidized-reduced glycopeptides were subjected to mild acid hydrolysis, a mixture of N-acetylgalactosaminyl and N-acetylgalactosaminyl gluceraldehyde was obtained. This indicates that N-acetylgalactosamine and N-acetylgalactosaminyl erythritol or glycerol on Smith degradation.

Although methylation and enzymatic hydrolysis showed N-acetylgalactosamine to be located terminally, it was resistant to periodate oxidation. The reason for this discrepancy is not understood at present. The possibility of incomplete oxidation due to mild experimental conditions used for oxidation or due to intersugar hemiacetal formation was discounted by carrying out oxidation reaction under more severe conditions or by the reoxidation of the oxidized-reduced oligosaccharide. Similarly, the probability of any alkali-labile substituent at C-3 or C-4 of N-acetylgalactosamine was ruled out by treatment of the glycopeptides with 2 M NH4OH prior to periodate oxidation. Such a substituent could get hydrolyzed during methylation, and thus, erroneously show N-acetylgalactosamine as terminally located. The structure of the peripheral parts of the oLH carbohydrate was elucidated by the low molecular weight fraction obtained from the acetylation products of the oligosaccharide after reduction with NaBH4. This fraction was made...
up of N-acetylglalactosamine, N-acetylglucosamine, and man- 
nitol in a ratio of 1:1:2. Since [1-3H]mannitol was the only 
reduced sugar, mannose must be present at the reducing end 
of the oligosaccharide(s). From the relative proportion of 
the three monosaccharides and paper chromatographic mobility 
in three solvent systems known to resolve di- and trisaccha- 
drides (48), the low molecular weight fraction appears to be 
a mixture of GlcNAc-β1,2-Man-H₂ and GalNAc-β1,2-Man-H₂.

The methylation analysis showed N-acetylglucosamine to 
be substituted at C-4. The substituent was acid-labile since 
methylation carried out after treatment of the glycopeptides 
with mild acid showed the presence of terminal N-acetylga-
lactosamine. The substituent was identified as sulfate ester by 
the isolation of 2,5-anhydrotalitol 4-sulfate from the deami-
nation mixture of the oLH-oligosaccharide. Subsequent to our 
preliminary communication (11, 12), Parsons and Pierce (49) 
reported the presence of two sulfate ester groups, one each of 
C-4 of N-acetylglactosamine and N-acetylglucosamine. The 
presence of sulfate ester group on the nonreducing terminal 
of N-acetylglucosamine cannot be reconciled with our data 
since the methylation analyses of all three glycopeptides showed 
the presence of 1 residue of terminal N-acetylglucosamine. 
Also, while the deamination studies of the oligosaccharide 
yielded 2,5-anhydrotalitol 4-sulfate, no sulfated 2,5-anhydro-
mannitol was detected in the deamination products of oLH-
oligosaccharide. The possibility of a sulfate diester, however, 
involving C-4 hydroxyl groups of N-acetylglucosamine and 
N-acetylglactosamine on the two oligosaccharide branches of 
the carbohydrate unit cannot be ruled out. The presence of 
such a group can explain the resistance of the N-acetylgluc-
oseamine to periodate oxidation. It may be pointed out that a 
fraction of N-acetylglucosamine was substituted by galactose 
in oLH and not in bLH (11, 12).

The evidence in support of the presence of N-acetylglac-
tosamine on the 1,6-mannosyl branch while N-acetylgluco-
samine on 1,3-mannosyl branch was derived from acetylation 
studies. The acetylation of the oligosaccharide gave two large 
molecular weight fractions GlcNAcβ1,2Man-α1,3Man-α1,4Glc-
NAcβ1,2GlcNAc-H₂ and GlcNAcβ1,2Man-α1,3Man-α1,4Glc-
NAcβ1,2GlcNAc-H₂, the latter lacking N-acetylglactosamine 
4-sulfate and fucose and possibly some galactose while the 
high mannose type (50).

The direct evidence for the presence of a trimannose unit 
in the core of the carbohydrate structure comes from deamina-
tion studies. The deamination of the oligosaccharide yielded 
a tetrasaccharide, the structure of which, established by methy-
lolation, periodate oxidation, and hydrolysis with exoglyco-
dases, was Manα1,6(Mannα1,3Man)β1,2Mana1,6Man-H₂. The 
trimannose unit was thus attached to the penultimate N-ac-
etylglucosamine at the reducing end. The structure of the 
core trisaccharide β1,4-mannosyl chitobiose was established 
after its isolation by NaOH + NaBH₄ treatment of the Smith 
degradation product of oLH. The β-mannosidase hydrolysis 
of the trisaccharide resulted in the formation of chitobiotol.

The sugar involved in the carbohydrate-protein linkage was 
obviously N-acetylglucosamine since N-acetylglucosaminotidol 
present at the reducing end of the oligosaccharide was formed 
by the cleavage of protein carbohydrate linkage with NaOH + 
NaBH₄. The location of fucose to the C-6 position of the 
innermost N-acetylglucosaminyl residue was assigned on the 
basis of methylation data. The presence of 2-deoxy-3-O-
methyl-2-N-methylacetamido-n-glucose, and its subsequent 
disappearance after partial acid hydrolysis suggested the pre-

cence of fucose on the innermost N-acetylglucosamine.

This was further confirmed by the isolation of fucosyl-2,5-anhydro-
mannitol from the deamination products. Galactose was 
linked to N-acetylglucosamine on the basis of the formation 
of galactosyl-2,5-anhydromannitol on deamination.

Finally, the anomeric linkages were established by the 
enzymatic hydrolysis with specific glycosidases of the various 
fragments of the structure obtained by various chemical meth-
ods such as N-acetylglucosaminyl glyceraldehyde and N-
acetylglucosaminyl glyceraldehyde from the hydrolsyate of 
the periodate oxidized-reduced glycopeptides, GalNAc-Man-
H₂ and GlcNAc-Man-H₂ from the acetylation products. 
Manβ1,6-GlcNAc-Manβ1,6-GlcNAc-Man-α1,3Manβ1,6Mana1,6-
Man-H₂ from deamination products and Man-GlcNAc-GlcNAc-
H₂ by NaOH-NaBH₄ treatment of the periodate oxidized-reduced 
glycopeptide after mild acid hydrolysis.

The presence of a sulfate ester group at the nonreducing 
terminal N-acetylglactosamine is a novel feature of this 
structure which has not been observed so far in any other 
animal glycoprotein. Only recently, Prehn et al. (51) have 
reported the existence of ethylidene group formed between 
aractaldehyde and C-4 and C-6 hydroxyl group of the nonre-
ducing terminal galactose of a glycoprotein from paramyxo-

virus-infected bovine kidney cell culture. The role of the 
substituent and N-acetylglactosamine in the synthesis and 
secretion of the hormone, its plasma survival and cellular 
uptake and, last not least, in its function are some of the 
important questions which still remain to be answered.

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